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Impact of oral meloxicam on circulating physiological biomarkers of stress and inflammation in beef steers after long-distance transportation

Abstract

Transportation stress can result in significant economic losses to producers due to decreased animal productivity and increased medication costs associated with sickness such as bovine respiratory disease (BRD). Meloxicam (MEL) provides pain relief and anti-inflammatory effects in cattle for several days after a single oral treatment. Our hypothesis was that MEL administration before shipping would reduce the impact of long-distance transportation on circulating physiological biomarkers of stress and inflammation in beef steers. Ninety-seven beef steers were blood sampled for baseline biomarker determination and then randomly assigned to receive either 1 mg/kg MEL ($n = 49$) or a placebo (CONT; $n = 48$) per os before a 1,316-km transportation event lasting approximately 16 h. Calves were then blood sampled on arrival and 5 d later. Changes in the hemogram, circulating plasma proteins, total carbon dioxide (TCO_2), fibrinogen, substance P (SP), cortisol, haptoglobin (Hp)-matrix metalloproteinase-9 (MMP-9) complexes, and tumor necrosis factor α (TNF α) between treatments over time were compared using a mixed effects model with statistical significance designated as $P < 0.05$. Analysis of covariance was conducted to assess the relationship between circulating MEL concentrations and biomarker changes over time. An increase in neutrophil, platelet, monocyte, white blood cell, and red blood cell counts occurred after transportation ($P < 0.0001$) and a decrease in lymphocyte count were observed ($P < 0.0001$). Meloxicam treatment reduced the stress-induced neutrophilia ($P = 0.0072$) and circulating monocyte count ($P = 0.013$) on arrival. Mean corpuscle hemoglobin ($P = 0.05$), mean corpuscle volume ($P = 0.05$), and lymphocyte count ($P = 0.05$) were also greater in the CONT calves compared with MEL calves after transportation. Furthermore, Hp-MMP-9 complexes, TCO_2 , TNF α , plasma proteins, and SP increased and cortisol decreased after shipping ($P < 0.01$). Meloxicam treatment tended to reduce serum cortisol concentrations ($P = 0.08$) and there was evidence of a time \times treatment interaction ($P = 0.04$). An inverse relationship between plasma MEL concentrations and circulation cortisol concentrations ($P = 0.002$) and neutrophil ($P = 0.04$) and basophil counts ($P = 0.03$) was also observed. The results suggest that MEL administration may reduce the impact of long-distance transportation on circulating physiological biomarkers of stress and inflammation in beef calves.

Keywords

inflammation, meloxicam, steers, stress, transportation

Disciplines

Large or Food Animal and Equine Medicine | Other Veterinary Medicine | Veterinary Toxicology and Pharmacology

Comments

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Impact of oral meloxicam on circulating physiological biomarkers of stress and inflammation in beef steers after long-distance transportation¹

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ABSTRACT: Transportation stress can result in significant economic losses to producers due to decreased animal productivity and increased medication costs associated with sickness such as bovine respiratory disease (BRD). Meloxicam (MEL) provides pain relief and anti-inflammatory effects in cattle for several days after a single oral treatment. Our hypothesis was that MEL administration before shipping would reduce the impact of long-distance transportation on circulating physiological biomarkers of stress and inflammation in beef steers. Ninety-seven beef steers were blood sampled for baseline biomarker determination and then randomly assigned to receive either 1 mg/kg MEL ($n = 49$) or a placebo (CONT; $n = 48$) per os before a 1,316-km transportation event lasting approximately 16 h. Calves were then blood sampled on arrival and 5 d later. Changes in the hemogram, circulating plasma proteins, total carbon dioxide (TCO₂), fibrinogen, substance P (SP), cortisol, haptoglobin (Hp)-matrix metalloproteinase-9 (MMP-9) complexes, and tumor necrosis factor α (TNF α) between treatments over time were compared using a mixed effects model with statistical significance designated as $P < 0.05$. Analysis of covariance was conducted to

assess the relationship between circulating MEL concentrations and biomarker changes over time. An increase in neutrophil, platelet, monocyte, white blood cell, and red blood cell counts occurred after transportation ($P < 0.0001$) and a decrease in lymphocyte count were observed ($P < 0.0001$). Meloxicam treatment reduced the stress-induced neutrophilia ($P = 0.0072$) and circulating monocyte count ($P = 0.013$) on arrival. Mean corpuscle hemoglobin ($P = 0.05$), mean corpuscle volume ($P = 0.05$), and lymphocyte count ($P = 0.05$) were also greater in the CONT calves compared with MEL calves after transportation. Furthermore, Hp-MMP-9 complexes, TCO₂, TNF α , plasma proteins, and SP increased and cortisol decreased after shipping ($P < 0.01$). Meloxicam treatment tended to reduce serum cortisol concentrations ($P = 0.08$) and there was evidence of a time \times treatment interaction ($P = 0.04$). An inverse relationship between plasma MEL concentrations and circulation cortisol concentrations ($P = 0.002$) and neutrophil ($P = 0.04$) and basophil counts ($P = 0.03$) was also observed. The results suggest that MEL administration may reduce the impact of long-distance transportation on circulating physiological biomarkers of stress and inflammation in beef calves.

Key words: inflammation, meloxicam, steers, stress, transportation

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INTRODUCTION

Transportation of calves intended for beef production is a common practice. In the United States, at least 65% of calves are shipped to feedlots in Texas, Kansas, Nebraska, and Colorado, usually by truck, due to a geographical separation between calf rearing and finishing facilities (Swanson and Morrow-Tesch, 2001;

U.S Food and Drug Administration, 1994). Immunosuppression after transportation predisposes calves to developing bovine respiratory disease (**BRD**), which commonly occurs in the first 45 d of arrival and is associated with 65 to 80% morbidity and 35 to 55% mortality (Cernicchiaro et al., 2012; Edwards, 1996; Speer et al., 2001). Bovine respiratory disease is estimated to cost the beef industry US\$500 million annually (Miles, 2009). Therefore, even a modest reduction in stress after transportation could have a significant economic benefit for livestock producers (Speer et al., 2001).

Transportation is associated with an increase in circulating pro-inflammatory cytokines in cattle leading to the initiation of an inflammatory reaction (Arthington et al., 2003). Previous studies support the hypothesis that extended exposure to a nonsteroidal anti-inflammatory drug (**NSAID**) may reduce stress and improve performance after castration and dehorning (Baldrige et al., 2011; Coetzee et al., 2012b). Meloxicam (**MEL**) is an NSAID approved in Canada for pain relief in calves after disbudding and is used as an anti-inflammatory for dogs in the United States. Meloxicam was found to reduce the incidence of BRD in calves after castration when administered on arrival at the feedlot (Coetzee et al., 2011). However, a similar effect was not observed in calves received as steers. We hypothesized that MEL administration to beef steers before shipping may be effective at reducing the impact of transportation on stress biomarkers. The objective of this study was to assess stress biomarkers and inflammatory mediators in beef steers that received oral MEL or a placebo (**CONT**) before long-distance transportation.

MATERIALS AND METHODS

Before the initiation of this experiment, all animal use, handling, and sampling techniques described were approved by the Iowa State University Animal Care and Use Committee (protocol number 5-12-7355-B).

Animals, Housing, Treatment Allocation, and Shipment

This study was a randomized complete block design with cattle assigned to receive either oral MEL or a whey protein placebo before shipment based on bodyweight (Fig. 1). Ninety-seven medium-to-large frame, horned and polled, Brahman and Angus \times Brahman crossbred steers aged 15 to 17 mo, weighing 201 to 465 kg, were procured from the Brown Loam Experiment Station at the Mississippi State University research farm near Raymond, MS, after a 32 wk backgrounding period. All calves received preweaning vaccinations for infectious bovine rhinotracheitis, bovine viral diarrhea types I and

II, parainfluenza-3 virus, bovine respiratory syncytial virus (Pyramid 5; Boehringer Ingelheim, St. Joseph, MO), clostridial organisms (Ultra bac 7; Pfizer Animal Health, Madison, NJ), and *Mannheimia haemolytica* (One Shot; Pfizer Animal Health) and were dewormed at the facility with eprinomectin (Eprinex; Merial, Duluth, GA). Post-weaning vaccinations consisted of a booster for all pathogens stated before the start of the backgrounding period.

Following weaning in October 2011, calves were held for 60 d in the Brown Loam Research Facility in Raymond, MS, feedlot. Calves were initially fed a diet of soy hull pellets at 2% of their BW followed by free choice hay and 20% crude protein molasses. Animals were then maintained on ryegrass pastures until late June when they were allowed access to crabgrass and Bermuda grass pastures before shipping. If pasture was limited at any point in time, hay was provided. The hay in both instances contained mixed grasses but primarily consisted of Bermuda grass.

Baseline blood sample collection occurred at timepoint 0, which was immediately followed by administration of MEL or the CONT. Calves were ranked by BW and assigned to either a MEL- or CONT-treated group by a randomization table generated using Excel (Microsoft Corp., Redmond, WA). To facilitate dose determination and to minimize disruption during processing, calves were subdivided into 3 groups based on weights determined at study enrollment. These groups were as follows: light (201–314 kg), medium (319–370 kg), and heavy (380–465 kg) weight. At time zero (**0 h**) MEL tablets (MEL tablets United States pharmacopoeia 15 mg [United States pharmacopoeia 0378-1089-01], lot number 3032625; Mylan Pharmaceuticals Inc., Morgantown, WV) were administered to the 3 groups so that the average weight per group resulted in a mean dose of 1 mg MEL/kg BW. The doses were calculated using pretreatment BW from the previous week in addition to adjusted weight gain during that week. When weighed, no restrictions to food or water were implemented. The light group ($n = 31$) received an actual dose of 0.9 to 1.4 mg/kg BW. The medium group ($n = 33$) received an actual dose of 0.89 to 1.0 mg/kg BW, and the heavy group ($n = 33$) received an actual dose of 0.90 to 1.1 mg/kg BW. Calves in the CONT-treated group received an equivalent dose of whey protein supplement product (Health-watchers, Inc., Bohemia, NY), a pharmaceutically inactive excipient used in the manufacture of MEL tablets. Both treatments were administered in gelatin capsule boluses (lot number 2634; Torpac, Fairfield, NJ). Whey protein was also added as filler to the capsules containing MEL tablets so that operators remained masked to treatment group.

After treatment, calves were shipped approximately 1,316 km from Raymond, MS, to a feedlot facility near Tabor, IA, in 2 truckloads carrying 48 and 49 calves, respectively. Loading was coordinated for the trucks to arrive at the 24 h timepoint. Calves were blood sampled in Iowa on

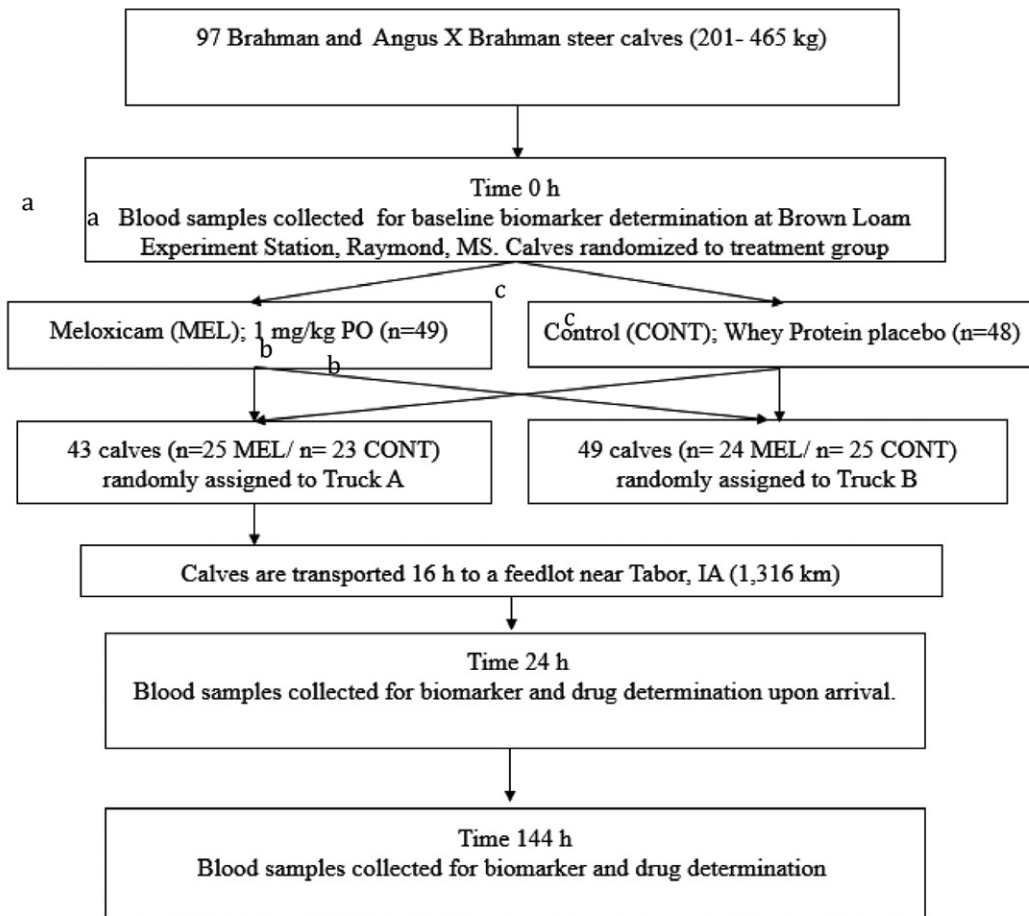


Figure 1. Flow chart outlining the timeline of study events that occurred at timepoints 0, 24, and 144 h. Treatment with either meloxicam at 1 mg/kg or a placebo was administered at time 0 h after baseline blood sample collection. Follow-up blood sample collection for determination of biomarkers and drug concentrations occurred after a 16 h (1,316 km) transportation event and 120 h thereafter.

10 July 2012 after a 16-h transportation event that concluded approximately 24 h after baseline blood collection. Calves were then maintained in an open pen of 192 m² with an allowance of approximately 0.69 m of bunk space per animal and 1 water source per pen. All calves were housed in the same pen. Upon arrival calves were fed a typical receiving diet composed of dry corn, dry distiller's grain, corn silage, and a protein/vitamin/mineral supplement throughout the experiment. Feed and water were offered ad libitum. A final blood sample was collected at 5 d after arrival, 144 h after baseline sample collection at 0 h.

Collection of Samples

Blood samples were collected at 0, 24, and 144 h via direct puncture of the jugular vein to assess complete blood count (CBC) and to determine plasma concentrations of MEL, total carbon dioxide (TCO₂), haptoglobin (Hp)-matrix metalloproteinase-9 (MMP-9) complexes, tumor necrosis factor α (TNF α), cortisol, and substance P (SP). Blood samples were collected in 6 mL evacuated tubes that contained lithium heparin (Vacuette Heparin

tubes; Greiner Bio-One, Monroe, NC; MEL and cortisol concentrations), serum separator tubes (Vacuette SST tubes; Greiner Bio-One; TCO₂, TNF α , and Hp-MMP-9), or EDTA tubes (Vacuette K3 EDTA tubes; Greiner Bio-One; CBC and SP concentrations). Benzamidine hydrochloride (lot number D1510; Santa Cruz Bio Technology, Dallas, TX) was added to 1 of the EDTA tubes at 1 mM per mL of whole blood to reduce endogenous protease-associated degradation of SP in the samples. Blood samples were centrifuged for 15 min at 1,600 \times g; 22°C. All samples were processed within 30 min of collection. Immediately after centrifugation, plasma was harvested, placed in cryovials, and frozen at -70°C until analysis.

Plasma Meloxicam Analysis

Plasma concentrations of MEL were determined using high-pressure liquid chromatography (Surveyor MS Pump and Autosampler; Thermo Scientific, San Jose, CA) with mass spectrometry detection (TSQ Quantum Discovery MAX; Thermo Scientific) as previously described (Kreuder et al., 2012). The accuracy of the assay

for MEL in bovine plasma was $99 \pm 3\%$ of the actual concentration while the coefficient of variation was 5% determined on 4 sets of replicates for each of the following concentrations: 15, 150, and 1,500 ng/mL.

Cortisol Analysis

Plasma cortisol concentrations were determined by RIA using a cortisol kit (Siemens Coat-a-Count Kit; Siemens, Malvern, PA) according to the manufacturer instructions. The range of detection was from 3 to 1,000 ng/mL. The coefficient of variation for intra-assay variability was 10% and the interassay variability was calculated at <15%.

Substance P Analysis

Substance P concentrations were analyzed as described by Liu et al. (2008) using nonextracted plasma. Method validation using nonextracted plasma consisted of complete recovery ($\pm 15\%$) of a known SP concentration added to pooled baseline plasma. Plasma concentrations of SP were analyzed by RIA using double antibody system with a primary antibody vs. SP (1:60,000, lot number H-061-0, purchased from Phoenix Pharmaceuticals, Burlingame, CA) and ^{125}I -substance P (20,000 counts per minute) purchased from PerkinElmer Inc. (Waltham, MA). Ethylenediaminetetraacetic acid (13 mM) immunoassays have been used previously to determine SP concentrations and have been validated for use with bovine plasma (Coetzee et al., 2008; Theurer et al., 2013). The assay had a determination range of 5 to 160 pg/mL. The coefficient of variation for intra-assay variability was at 11% and the interassay variability was calculated at 4%.

Total Serum Carbon Dioxide Analysis

Total carbon dioxide concentration was analyzed using the serum collected from steers at 0 and 24 h. Samples were centrifuged immediately after collection and all analyses were completed within 48 h after collection to minimize the effect of sample handling on TCO_2 concentrations (Tinkler et al., 2012). The samples were analyzed using Nova 4, a total CO_2 analyzer (Nova Biomedical, Waltham, MA) following the manufacturer's instructions. Data analysis was completed through the use of a known carbon dioxide standard kit (Verichem Laboratories, Providence, RI).

Tumor Necrosis Factor α Analysis

Tumor necrosis factor α was analyzed using a bovine TNF α ELISA test that was previously validated by

Farney et al. (2011). The optical density was then measured at 450 and 550 nm using a plate reader (PowerWaveXS; BioTek Instruments Inc., Winooski, VT). The corrected difference values were fit to the standard curve by point-to-point regression. Values of detection ranged from 3.9 to 250 pg/mL with intra-assay and interassay critical variances of 14.1 and 15.9%, respectively.

Haptoglobin-Matrix Metalloproteinase-9 Analysis

An ELISA specific for bovine neutrophil Hp in complex with MMP-9 was performed as described previously (Bannikov et al., 2011) with minor alterations. Standard concentrations ranged from 1.78 to 456 ng/mL, which represent the upper and lower limits of quantitation of the assay, respectively. The concentration of Hp-MMP-9 was determined using the linear portion of the equation of the line described by absorbance of the calibrators at 450 nm and the known concentration of these calibrators. Any sample remaining outside of the range of the standard curve (>456 ng/mL) were further diluted and reanalyzed. Between-plate variability of calibrators from 5 plates was less than 3% (median = 1.8%; range 0.98–2.7%). The average correlation coefficient determined by linear regression of the absorbance versus concentration of the calibrators was 0.91 (range 0.85 to 0.95). The analytical sensitivity of the assay was 3.5 ng/mL.

Clinical Pathology Analysis

The Iowa State University Clinical Pathology Laboratory determined the CBC using the ADVIA 120 analyzer (Siemens) using a program specific for bovine blood. This automated process measures total white blood cell count, white blood cell differential, red blood cell count, platelet counts, hemoglobin, fibrinogen, mean corpuscle volume (MCV), mean corpuscle hemoglobin concentration (MCHC), mean corpuscle hemoglobin (MCH), red blood cell distribution width (RDW), and hematocrit.

Statistical Analysis

The statistical analysis of the study was performed using SAS (version 9.2; SAS Inst. Inc., Cary, NC). Responses were analyzed using linear mixed models with repeated measures. Animal was the subject of repeated measures whereas truck was included in the model as a random effect. Two structures of fixed effects were considered. One is ANOVA structure with treatment group, time, and their interaction; the other is an analysis of covariance type with MEL concentration (continuous) and time (categorical) included in the model. Furthermore, matrix metalloproteinase (Hp-MMP-9) values were analyzed following quantification of Hp-MMP-9 as ei-

ther having the presence (“yes”) or absence (“no”) of the protein in measurable quantities. A mixed effect logistic regression model that contained repeated measures was used to describe the data.

Model assumptions were considered to be appropriately met based on diagnostics conducted on studentized residuals. Estimated least square means and corresponding standard errors, or 95% confidence intervals, are presented. A significant difference was considered to be present when $P \leq 0.05$, and a marginal difference was considered to exist if $0.05 < P \leq 0.10$. Relevant pairwise comparisons were conducted when the significance of the interaction term was $P \leq 0.10$ using Tukey-Kramer or Bonferroni adjustments, as appropriate in each case, to avoid inflation of Type I error rate due to multiple comparisons.

RESULTS AND DISCUSSION

Meloxicam is an NSAID of the oxicam class that is approved in the European Union and Canada for adjunctive therapy of acute respiratory disease, diarrhea, and acute mastitis and the alleviation of pain associated with disbudding in calves when administered at 0.5 mg/kg intravenously or subcutaneously (EMA, 2009). Meloxicam administered to cattle by any route constitutes extra-label drug use (ELDU) because there are currently no analgesic drugs specifically approved to provide pain or stress relief in livestock in the United States (Smith and Modric, 2013). Under the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA), ELDU is permitted only under veterinary supervision “when the health of an animal is threatened, or suffering or death may result from failure to treat” provided specific conditions are met (U.S. Food and Drug Administration, 1994, 530.2). In the absence of U.S. Food and Drug Administration–approved analgesic compounds in food animals, use of oral MEL tablets for alleviation of pain or stress in cattle can be considered under AMDUCA. To our knowledge, this is the first published report evaluating the effect of a long-acting NSAID on the stress response in cattle after long-distance transportation. Further studies are needed to assess if this translates to improved health and performance on arrival at the feedlot.

Plasma Meloxicam Concentrations

None of the calves had detectable plasma MEL concentrations in samples collected at the baseline timepoint. Mean (\pm SEM) plasma MEL concentrations in MEL-treated calves ranged from $1,868 \pm 92.16$ ng/mL on arrival at the feedlot (24 h after dosing) to 38.38 ± 6.18 ng/mL at 144 h after dosing. There was no differ-

ence in plasma MEL concentrations between the 3 different weight categories of calves that were used for dose determination ($P = 0.90$). There were also no MEL detected in calves assigned to the CONT group.

The pharmacokinetics of MEL after oral administration at 1 mg/kg indicate that a peak plasma concentration of approximately 3 μ g/mL occurs at around 12 h after administration with an elimination half-life ($T_{1/2}$) of about 28 h (Coetzee et al., 2009, 2011). In the present study, the mean MEL concentration determined at 24 h after oral administration was less than the peak concentration previously reported. However, the mean concentration of MEL recorded at 144 h represents a $T_{1/2}$ of approximately 24 h and is consistent with the level expected after approximately 5 to 6 plasma elimination half-lives. Taken together the results of this study suggest that MEL administered at 1 mg/kg provided circulating MEL concentrations for up to 5 d after oral administration.

Body Weight

Calves experienced a characteristic decrease in BW after the transportation event known as “shrink.” This weight loss was reported as a time effect ($P < 0.001$) with no treatment effect observed between groups ($P = 0.67$; Fig. 2). Furthermore, there was no association between plasma MEL concentrations and shrink ($P = 0.78$; Table 1). These results are consistent with previously reported studies evaluating weight loss following transportation from a North American review on animal welfare (Schwartzkopf-Genswein et al., 2012). The observed weight loss in this study is consistent with previous reports in the literature. However, the calves in our study showed an 8% loss in BW whereas Arthington et al. (2003) reported a 2 to 3% loss in BW after a 3-h transportation event and Buckham Sporer et al. (2008) reported shrinkage of 10% in calves (approximately 230 kg) transported for over 9 h. This supports the hypothesis that percentage of weight loss during transportation is likely associated with the duration of the transportation event as was previously suggested (Cernicchiaro et al., 2012).

Cortisol

Serum cortisol concentrations were affected by time ($P < 0.0001$) in both the MEL and CONT groups. The concentration of cortisol decreased from baseline to 24 h after transportation and increased marginally at the 144-h sample collection (Fig. 3). The present study provided evidence of a time \times treatment interaction on cortisol response ($P = 0.04$) and an inverse relationship between circulating cortisol concentrations and MEL concentrations ($P = 0.0017$; Table 1).

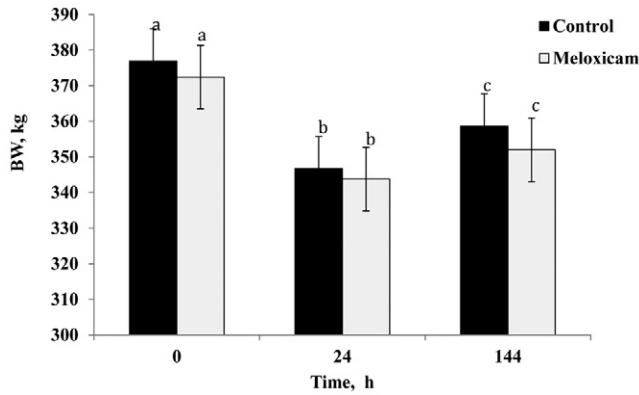


Figure 2. Mean \pm SE for BW change (kg) after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

The decrease in cortisol observation contradicts previous published reports that demonstrate an increase in cortisol ranging from 5.8% in 2 to 3 wk mixed breed calves transported for 8 to 24 h in duration (Knowles et al., 1997) to 311% in 4 to 6 mo old dairy calves transported for 4 h (Murata et al., 1987). Elevated cortisol concentrations in serum are maintained after exogenous ACTH stimulation for approximately 2 h (Lay et al., 1996). The longer travel time in the present study may have allowed calves to become acclimated to their surroundings, thus reducing blood cortisol levels while on the truck in transit. Moreover, the sample obtained at 24 h may not have coincided with peak cortisol concentrations following transportation.

The observed time \times treatment effect was likely due to the combined anti-inflammatory and analgesic effect of NSAID in addition to a proposed direct modulation of ACTH release resulting in a reduction in circulating cortisol concentrations (Watson et al., 2009). Recently, Geary (2012) reported that circulating cortisol concentration in cows receiving the NSAID flunixin meglumine was numerically less than control cows after injection of ACTH to mimic transportation stress. It is likely that this study, which comprised only 20 animals, lacked sufficient statistical power to detect a significant difference between groups. Taken together, the relationship between NSAID administration and stress reduction after transportation warrants further investigation to determine if this results in improved health and performance of calves after long-distance transportation.

Haptoglobin-Matrix Metalloproteinase-9 Complexes

The majority of the serum samples contained <15 ng/mL Hp-MMP-9 complexes (253/288 samples). Therefore, in addition to conducting a repeated measures analysis on the positive results, we also chose to classify

Table 1. Intercept and slope of chemistry biomarkers and bodyweight change with SE for the correlation between meloxicam (MEL) plasma concentration and the outcome variables

Parameter	Intercept	log MEL		<i>P</i> -value
		Slope gradient estimate	SE	
Bodyweight, kg	354.34	0.08	0.30	0.78
Log cortisol, ng/mL	37.19	−1.51	0.47	0.0017
Substance P, pg/mL	26.06	0.16	0.78	0.89
Plasma protein, g/dL	6.96	−0.01	0.01	0.32
Fibrinogen, mg/dL	454.96	3.03	3.76	0.42
Haptoglobin-matrix metalloproteinase-9 complexes, ng/mL	1.42	−0.02	0.05	0.74
Total carbon dioxide, mM	24.91	−0.01	0.06	0.90
Tumor necrosis factor-alpha, pg/mL	168.05	0.09	1.91	0.96

samples as positive or negative based on the cutoff of <15 ng/mL (negative) and >15 ng/mL (positive). Overall 2.1% of the calves were positive at first sampling time, 15.6% were positive at 24 h after arrival, and 18.9% were positive at 4 d after arrival. However, using this analysis there was no effect of treatment ($P = 0.97$), time ($P = 0.88$), or time \times treatment interaction ($P = 0.59$).

In the calves that did demonstrate quantifiable increases in Hp-MMP-9 complexes, a time effect ($P = 0.0061$) was observed in both treatment groups (Fig. 4) but no treatment effect ($P = 0.88$) was observed. There was also no evidence of a relationship between MEL concentrations and log Hp-MMP-9 ($P = 0.74$; Table 1).

Matrix metalloproteinases regulate the degradation of extracellular matrix protein components and modulates cytokine activation through in vivo cleavage. Bannikov et al. (2007) reported that neutrophil MMP-9 is covalently complexed with neutrophil synthesized Hp suggesting that this is a potentially useful biomarker indicating activation and degranulation of neutrophils (Theurer et al., 2013). Increases in plasma Hp-MMP-9 concentration over time are consistent with other studies reporting significantly elevated plasma Hp-MMP-9 concentrations in cattle with acute inflammation (Bannikov et al., 2011). A number of disorders commonly diagnosed in cattle are characterized by a significant mobilization and activation of neutrophils (Slocumbe et al., 1985). The present study documents for the first time that plasma concentrations of Hp-MMP-9 are associated with physiologic alterations during transportation.

Fibrinogen

Circulating fibrinogen concentration was not significantly affected by transportation ($P = 0.17$) or MEL

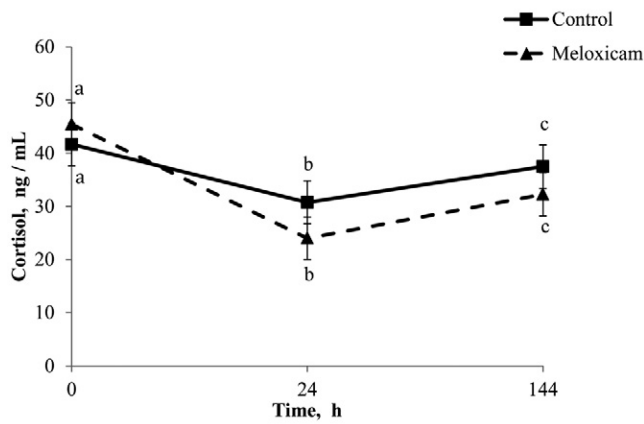


Figure 3. Mean \pm SE for serum cortisol concentrations after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

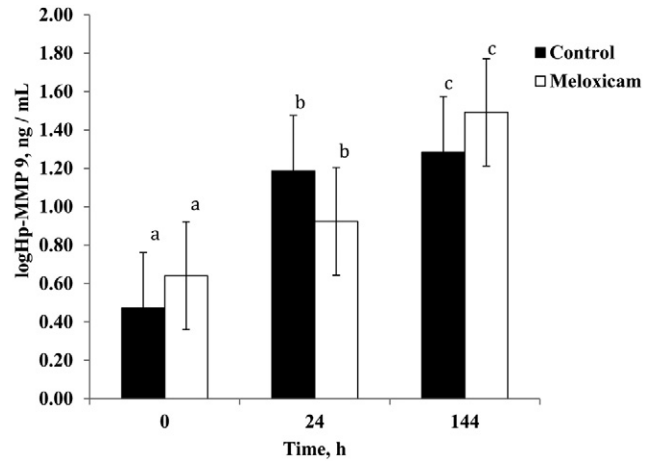


Figure 4. Mean \pm SE for plasma log haptoglobin (Hp)-matrix metalloproteinase-9 (MMP-9) complexes concentrations after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

administration ($P = 0.41$; Table 2). There was also no association between MEL concentrations and fibrinogen ($P = 0.42$; Table 1). Fibrinogen maintains homeostasis by providing a substrate for fibrin formation and tissue repair (Murata et al., 2004). Fibrinogen results for cattle during transportation remain equivocal. A 133% increase in fibrinogen was reported in a study with 32 calves that were transported for 3 h (Arthington et al., 2003). Conversely, a 28% reduction in fibrinogen over 48 h after transportation was reported in a study involving 36 calves transported for 9 h (Buckham-Sporer et al., 2008). Therefore the usefulness of fibrinogen as a biomarker of inflammation after transportation requires further investigation.

Plasma Protein

Plasma protein concentrations increased over time after transportation ($P < 0.0001$), most notably at the 24-h sampling timepoint ($P < 0.001$) as illustrated in Table 1. Plasma protein was unaffected by treatment ($P = 0.32$) and there was no association between plasma MEL concentrations and plasma protein ($P = 0.32$; Table 1).

Hyperproteinemia was anticipated after transportation as a result of dehydration due to inadequate water intake and water loss during shipping. This observation is in agreement with previous reports (Parker et al., 2003; Earley et al., 2006a; Earley and O'Riordan, 2006b). However, another study reported a decrease in plasma protein after a 9-h transportation event (Buckham Sporer et al., 2008). This was attributed to altered protein metabolism associated with transportation or differences in the nutritional status of animals immediately before transportation. It was also reported that breed may influence plasma protein concentrations but these effects could not be established in the present study.

Tumor Necrosis Factor α

A significant increase in circulating TNF α concentrations was observed after transportation ($P < 0.001$; Fig. 5). However, there was no effect of MEL administration on TNF α concentration ($P = 0.79$). There was also no association between plasma MEL concentrations and TNF α concentrations ($P = 0.96$; Table 1).

Table 2. Comparison between the least squares (LS) means \pm SE serum chemistry biomarkers at 0, 24, and 144 h after treatment (trt) with 1 mg/kg meloxicam (MEL) or a placebo (CONT) before transportation

Parameter	Normal range	Trt group	Time, h			P-values		
			0	24	144	Trt	Time	Time \times trt
			LS means \pm SE	LS means \pm SE	LS means \pm SE			
Plasma protein, g/dL	6.9–7.7	CONT	7.02 ^a \pm 0.07	7.40 ^b \pm 0.07	7.00 ^a \pm 0.07	0.32	<0.0001	0.18
		MEL	7.02 ^a \pm 0.07	7.32 ^b \pm 0.07	6.88 ^b \pm 0.07			
Total carbon dioxide, mM		CONT	22.77 ^a \pm 0.33	24.98 ^b \pm 0.34		0.51	<0.0001	0.77
		MEL	22.43 ^a \pm 0.33	24.81 ^b \pm 0.33				
Fibrinogen, mg/dL	100–500	CONT	438.11 \pm 24.68	462.39 \pm 24.68	483.8 \pm 25.09	0.41	0.173	0.30
		MEL	426.23 \pm 24.30	477.62 \pm 24.12	433.85 \pm 25.54			

^{a,b}Different superscripts indicate significant differences between timepoints in a row after transportation ($P < 0.05$).

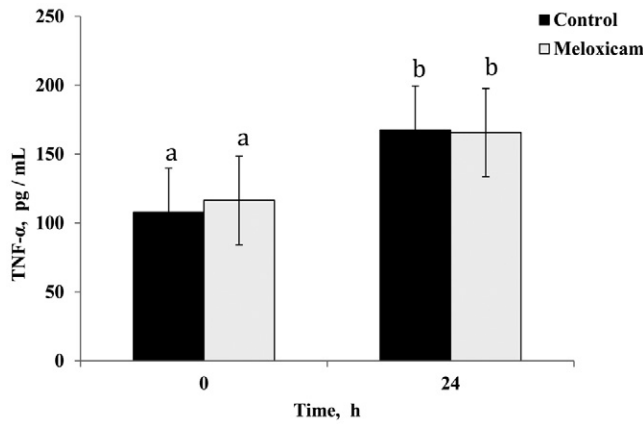


Figure 5. Mean \pm SE for serum tumor necrosis factor- α (TNF α) concentrations after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

Cytokines are signaling proteins produced primarily by macrophages in response to infection, tissue injury, or stress. Elevated levels of TNF α increase vascular permeability of endothelial cells, which allows for the increased entry of immunoglobulin, complement, and other cells to damaged tissue (Janeway et al., 2012). An increase in cytokine production after transportation was anticipated based on a recent publication that reported a 40% increase in TNF α at 48 h after a 4-h transit event (Hulbert et al., 2011). Although a recent study indicated a reduction of TNF α in calves with BRD treated with MEL and oxytetracycline, a treatment effect was not observed in the present study (Bednarek et al., 2003). The direct mechanism behind this effect is poorly understood and requires further investigation.

Substance P

An increase in plasma SP concentrations was observed after transportation ($P < 0.0026$; Fig. 6), but there was no treatment effect ($P = 0.13$) or time \times treatment interaction ($P = 0.45$). There was also no association between plasma MEL concentrations and SP ($P = 0.89$; Table 1).

Substance P is an 11-amino acid neuropeptide associated with areas of the neuroaxis involved in the integration of pain, stress, and anxiety (Devane, 2001; Coetzee et al., 2008). The present study supports the results of a recently reported 46% SP increase following 4 h of transportation (Theurer et al., 2013). Although an inverse linear relationship of MEL and SP has been recently reported in a dehorning study (Coetzee et al., 2012), MEL administration did not reduce SP in the present study. The absence of an effect could be associated with a reduced magnitude of SP release after transportation compared with dehorning.

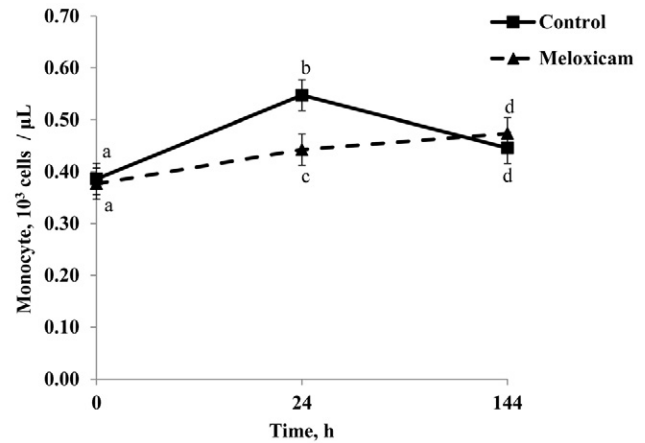


Figure 6. Mean \pm SE for plasma substance P concentrations after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

A wide variety of physical and psychological stressors can stimulate release of SP. Pulmonary exposure to diesel exhaust fumes, ozone, allergens, carbon dioxide, and cigarette smoke can elicit SP release in airways in rats (Wong et al., 2004a,b). The role of SP in the pathogenesis of BRD has yet to be elucidated. In experimental *M. haemolytica* infection in sheep there was strong immunoreactivity for SP on macrophages infiltrating into the inflamed areas on d 1 postinfection (Ramirez-Romero et al., 2001; Grubor et al., 2004). Furthermore, exposure of alveolar macrophages to SP resulted in enhanced phagocytosis and increased tumor necrosis factor production compared to nonstimulated controls (Rogers et al., 2006). An increase in vascular permeability in response to SP exposure has also been demonstrated (Ragsdale et al., 2008). Taken together these studies indicate that transportation increases SP release and that this may be associated with the development of pulmonary inflammation that may contribute to the pathogenesis of BRD. However, administration of an NSAID appeared to be ineffective at directly mitigating SP release after shipping in the present study.

Total Carbon Dioxide

There was an increase in TCO₂ during transit ($P < 0.0001$; Table 2) but there was no treatment effect ($P = 0.51$) or association between plasma MEL concentrations and TCO₂ ($P = 0.90$; Table 1).

An increase in TCO₂ after shipping has been previously reported as a result of a decrease in potassium, chloride, and water from sweating (Stockham et al., 2008). However, in a study investigating the partial pressure of carbon dioxide after transportation, no difference was reported for transported steers compared to control animals (Parker et al., 2003). Total carbon

Table 3. Comparison between the least squares (LS) means \pm SE of red blood cell parameter data at 0, 24, and 144 h after treatment (trt) with 1 mg/kg meloxicam (MEL) or a placebo (CONT) before transportation at 0 h, after transportation at 24 h, and at 144 h thereafter

Parameter	Normal range	Trt group	Time, h			P-values		
			0	24	144	Trt	Time	Time \times trt
			LS means \pm SE	LS means \pm SE	LS means \pm SE			
Red blood cell, 10^3 cells/ μ L	5.0–10.0	CONT	8.47 ^a \pm 0.14	8.59 ^a \pm 0.14	8.19 ^b \pm 0.14	0.13	<0.0001	0.38
		MEL	8.80 ^a \pm 0.14	8.79 ^a \pm 0.14	8.50 ^b \pm 0.14			
Hemoglobin, g/dL	8.0–15.0	CONT	11.75 ^a \pm 0.15	12.00 ^b \pm 0.15	11.27 ^c \pm 0.15	0.98	<0.0001	0.17
		MEL	11.83 ^a \pm 0.15	11.85 ^a \pm 0.15	11.33 ^b \pm 0.15			
Hematocrit, %	24.0–46.0	CONT	33.12 ^a \pm 0.43	33.94 ^b \pm 0.43	31.90 ^c \pm 0.44	1.00	<0.0001	0.20
		MEL	33.42 ^a \pm 0.42	33.52 ^a \pm 0.42	32.04 ^b \pm 0.43			
Mean corpuscle volume, fL	40.0–60.0	CONT	39.27 ^a \pm 0.45	39.69 ^b \pm 0.45	39.15 ^a \pm 0.45	0.05	<0.0001	0.20
		MEL	38.17 ^a \pm 0.44	38.33 ^a \pm 0.44	37.91 ^b \pm 0.44			
Mean corpuscle hemoglobin, Pg	11.0–17.0	CONT	13.95 ^a \pm 0.18	14.03 ^a \pm 0.18	13.83 ^b \pm 0.18	0.05	<0.0001	0.61
		MEL	13.51 ^{ab} \pm 0.18	13.55 ^a \pm 0.18	13.41 ^b \pm 0.18			
Mean corpuscle hemoglobin concentration, g/dL	30.0–36.0	CONT	35.52 \pm 0.16	35.35 \pm 0.16	35.35 \pm 0.17	0.87	0.46	0.70
		MEL	35.40 \pm 0.16	35.37 \pm 0.16	35.37 \pm 0.17			
Red blood cell distribution width, %	8.0–15.0	CONT	18.78 ^a \pm 0.27	18.42 ^{b,x} \pm 0.27	18.97 ^a \pm 0.27	0.14	<0.0001	0.07
		MEL	19.00 \pm 0.27	18.95 ^y \pm 0.27	19.20 \pm 0.27			

a,b,c Different superscripts indicate significant differences between timepoints in a row after transportation ($P < 0.05$).

x,y Different superscripts indicate significant differences between treatment groups in the related CONT and MEL columns after transportation ($P < 0.05$).

dioxide of serum or plasma provides a measure of acid–base balance due to metabolic changes. The increase in TCO₂ observed in the present study is likely indicative of either a metabolic or respiratory alkalosis (Dehkordi and Dehkordi, 2011). Metabolic alkalosis is most frequently associated with digestive disturbances in ruminants. Respiratory alkalosis is caused by hyperventilation, which may be stimulated by pulmonary disease or heat stress. Both types of alkalosis may therefore be present during long-distance transportation where animals are subjected to food deprivation and exposed to changes in environmental temperature.

Hematology

Time effects were observed for all hematological data ($P < 0.05$), except for basophil count ($P = 0.84$) and MCHC ($P = 0.45$; Table 3). Treatment effects were observed for total leukocyte counts ($P = 0.01$), MCV ($P = 0.05$), MCH ($P = 0.05$), and lymphocyte count ($P = 0.05$; Table 4). A time \times treatment interaction was evident for monocyte count ($P = 0.04$) and a trend towards an interaction was observed for RDW ($P = 0.07$) and neutrophil count ($P = 0.10$).

A stress leukogram, characterized by a mature neutrophilia, lymphopenia, and monocytosis, was observed in both groups following transportation. The administration of MEL appeared to attenuate the development of a stress leukogram as evidenced in the response variables discussed below.

A neutrophilia ($P < 0.0001$) observed across both groups after transportation (Fig. 7) is consistent with results from previous studies (Blecha et al., 1984; Buckingham Sporer et al., 2008; Burdick et al., 2011). The increase in the mean number of circulating neutrophils recorded in the CONT group on arrival at the feedlot was greater than in the MEL group ($P = 0.0072$; Table 4). Neutrophil count was also inversely proportional to plasma MEL concentrations providing further evidence of a treatment response ($P = 0.04$; Table 5).

A stress neutrophilia is the result of the corticosteroid-induced release of mature cells from the bone marrow and the reduced margination and migration of neutrophils out of the vasculature (Benschop et al., 1996; Jones et al., 2007). Several studies have determined the functional capacity of the immune response using in vitro tests after transport. Murata et al. (1987) observed that nitroblue tetrazolium reduction activity of neutrophils was initially reduced by 10% on arrival followed by a 36% increase at 4 h after transport. Similarly, Burdick et al. (2011) found that cattle had decreased phagocytic neutrophil intensity at 48 h posttransit and that calm bulls had more active neutrophils with greater phagocytosis and oxidative burst intensities to *Escherichia coli* than temperamental bulls at 96 h. These findings indicate that, although the relative number of neutrophils is increased after transportation, the functional capacity of these cells is decreased leading to immune compromise. The mitigation of the stress neutrophilia in the present study and the negative association between MEL concentrations and neutrophil count suggests that MEL may have reduced

Table 4. Comparison between the least squares (LS) means \pm SE of white blood cell parameter data at 0, 24, and 144 h after treatment (trt) with 1 mg/kg meloxicam (MEL) or a placebo (CONT) before transportation at 0 h, after transportation at 24 h, and at 144 h thereafter

Parameter	Normal range	Trt group	Time, h			P-values		
			0	24	144	Trt	Time	Time \times trt
			LS means \pm SE	LS means \pm SE	LS means \pm SE			
Platelet, 10^3 cells/ μ L	100–800	CONT	389.47 ^a \pm 23.25	392.62 ^a \pm 23.25	534.26 ^b \pm 23.57	0.81	<0.0001	0.14
		MEL	395.53 ^a \pm 22.80	409.78 ^a \pm 22.80	490.79 ^b \pm 23.75			
Neutrophil, 10^3 cells/ μ L	0.6–4.0	CONT	1.89 ^a \pm 0.23	3.88 ^{b,x} \pm 0.23	2.54 ^c \pm 0.24	0.10	<0.0001	0.10
		MEL	1.75 ^a \pm 0.23	3.18 ^{b,y} \pm 0.23	2.42 ^c \pm 0.24			
Lymphocyte, 10^3 cells/ μ L	2.5–7.5	CONT	7.32 ^{a,x} \pm 0.41	6.31 ^b \pm 0.41	5.69 ^c \pm 0.41	0.05	<0.0001	0.29
		MEL	6.43 ^{a,y} \pm 0.41	5.73 ^b \pm 0.41	5.20 ^c \pm 0.41			
Neutrophil:lymphocyte ratio, 10^3 cells/ μ L		CONT	4.90 ^a \pm 0.70	3.16 ^b \pm 0.70	2.41 ^b \pm 0.71	0.31	<0.0001	0.47
		MEL	4.51 ^a \pm 0.70	2.26 ^b \pm 0.69	2.51 ^b \pm 0.71			
Monocyte, 10^3 cells/ μ L	0.03–0.85	CONT	0.39 ^a \pm 0.03	0.55 ^{b,x} \pm 0.03	0.45 ^a \pm 0.03	0.27	0.0001	0.04
		MEL	0.38 ^a \pm 0.03	0.44 ^{ab,y} \pm 0.03	0.47 ^b \pm 0.03			
Basophils, 10^3 cells/ μ L	0–0.2	CONT	0.08 \pm 0.01	0.08 \pm 0.01	0.07 \pm 0.01	0.08	0.8336	0.11
		MEL	0.07 \pm 0.01	0.07 \pm 0.01	0.07 \pm 0.01			

^{a,b,c}Different superscripts indicate significant differences between timepoints in a row after transportation ($P < 0.05$).

^{x,y}Different superscripts indicate significant differences between treatment groups in the related CONT and MEL columns after transportation ($P < 0.05$).

some of the negative impacts of transportation stress on immune function. Further studies evaluating immune cell function are planned to characterize this effect further.

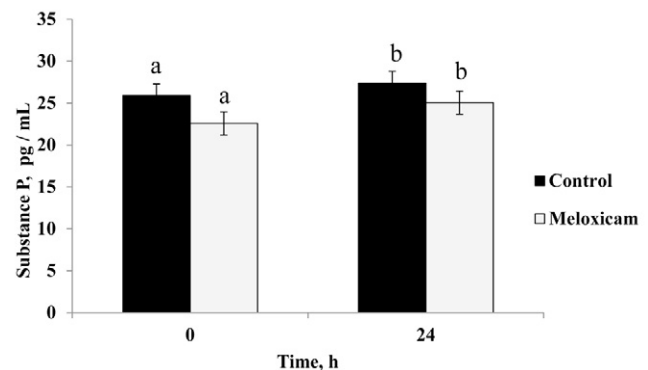
Lymphocyte counts decreased from before shipping to arrival in both the CONT ($P < 0.0001$) and MEL ($P < 0.0005$) groups (Table 4). A further reduction in lymphocyte count was observed between arrival and 120 h later in both the MEL ($P = 0.01$) and CONT ($P = 0.0001$) groups. The reason for the observed treatment effect of

MEL was likely due the greater lymphocyte count in the CONT calves before shipping because no time \times treatment interaction was evident (Fig. 8).

Exposure to corticosteroids causes redistribution of circulating lymphocytes into lymphoid compartments resulting in the development of a lymphopenia (Burton and Kehrl., 1996). Murata et al. (1987) reported a 90% decrease in T-lymphocyte numbers of 4- to 6-mo-old calves transported 4 h but no change in the number of B lymphocytes. Buckham Sporer et al. (2008) reported a 20% increase in leucocyte count from 4.5 to 14 h after initiation of a 9-h transportation event followed by a decrease ranging from 7 to 24% at 24 and 48 h, respectively. Hulbert et al. (2011) reported an 86% increase in the neutrophil:mononuclear cell ratio at 24 h posttransit. This observation is similar to the results of the present

Table 5. Intercept and slope of hematological biomarkers with SE for the correlation between meloxicam (MEL) plasma concentration and the outcome variables

Parameter	Intercept	Log MEL		P-value
		Slope gradient	estimate	
Basophils, 10^3 cells/ μ L	0.08	0.00	0.00	0.03
Neutrophil, 10^3 cells/ μ L	2.58	−0.07	0.03	0.04
Hemoglobin, g/dL	11.34	−0.03	0.02	0.06
Hematocrit, %	32.08	−0.09	0.05	0.07
Red blood cell distribution width, %	19.05	0.03	0.02	0.07
White blood cell, 10^3 cells/ μ L	9.05	−0.07	0.05	0.12
Red blood cell, 10^6 cells/ μ L	8.37	−0.02	0.01	0.20
Monocyte, 10^3 cells/ μ L	0.47	−0.01	0.00	0.27
Mean corpuscle volume, fL	38.52	−0.02	0.02	0.27
log mean platelet volume, fL	2.07	0.01	0.01	0.30
Neutrophil:lymphocyte ratio, 10^3 cells/ μ L	2.54	−0.05	0.06	0.42
Platelet, 10^3 cells/ μ L	508.83	2.78	3.60	0.44
Mean corpuscle hematocrit, g/dL	35.35	0.01	0.02	0.73
Mean corpuscle hemoglobin, pg	13.61	0.00	0.01	0.79
Lymphocyte, 10^3 cells/ μ L	5.45	−0.01	0.03	0.80

**Figure 7.** Mean \pm SE for circulating neutrophil counts after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

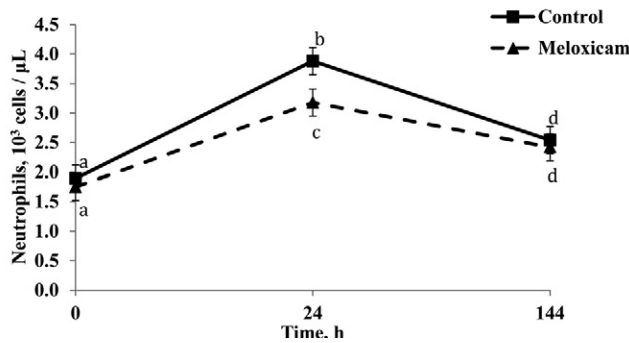


Figure 8. Mean \pm SE for circulating lymphocyte counts after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

study and is consistent with the development of a stress leukogram after transport.

Monocyte counts increased from before shipping to arrival in the CONT ($P < 0.0001$) but not the MEL ($P = 0.18$) group (Table 4; Fig. 9). Exposure to corticosteroids also causes mobilization of marginated monocytes within the vasculature resulting in the development of a monocytosis (Latimer et al., 2003). Burdick et al. (2011) reported an 8% increase in peripheral mononuclear cells after a 4-h transportation event ($P = 0.056$). This observation is similar to the results of the present study and is consistent with the development of a stress leukogram after transport. The concurrent mitigation of both the monocytosis and neutrophilia in the present study provides further support for the hypothesis that MEL reduced some of the negative impacts of transportation stress on immune function.

Basophilia in mammalian blood smears is seldom dramatic but an increase in circulating basophil count has been associated with stress in birds (Latimer and Prasse, 2003). In the present study there was no effect of time or treatment on basophil count (Table 4). However, there was a negative association between circulating MEL concentration and basophil count ($P = 0.03$; Table 5). To our knowledge there have been no studies that have specifically investigated the effect of transportation on basophil count. Given the absence of an overall effect of treatment and transportation despite a negative association with MEL, basophils appear to have limited utility in assessing stress in cattle.

A treatment effect was evident for both MCH ($P = 0.05$) and MCV ($P = 0.05$) with an observed decrease in MEL calves compared to CONT calves (Table 3). Although values remained in the laboratory's reference range for normal limits, this effect may be the result of decreased synthesis of PGE_2 associated with cyclooxygenase inhibition after NSAID administration. Prostaglandin E_2 has demonstrated both enhancement and maintenance of haemopoietic stem cell activity and increases in MCH and MCV are usually

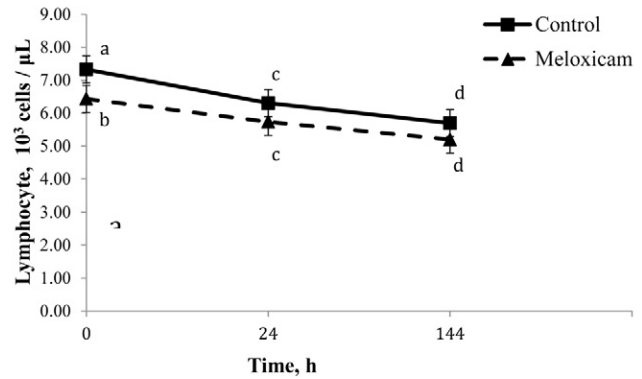


Figure 9. Mean \pm SE for circulating monocyte counts after treatment with 1 mg/kg meloxicam or a placebo at 0 h followed by a 16 h (1,316 km) transportation event. Letters that are the same indicate data points that are not significantly different ($P < 0.05$).

associated with hematopoiesis (North et al., 2007; Hoggatt et al., 2009). Therefore, compounds that decrease PGE_2 synthesis could potentially decrease stem cell activity resulting in a decreased MCH and MCV.

A time \times treatment interaction was evident in RDW ($P = 0.07$) with the distribution width being less in CONT calves compared with MEL calves ($P = 0.03$) on arrival (Table 3). There was no effect of time on RDW in the MEL-treated group. However, a weak positive association between MEL concentrations and RDW ($P = 0.07$) was observed.

Red blood cell distribution width is a measure of variation in the size of the red blood cells. It is a calculated parameter representing the coefficient of the variation of the MCV (Subhashree et al., 2013). It is therefore not surprising that a difference in RDW was observed in the present study because MCV was less in MEL-treated calves ($P = 0.05$). In a previous study, Fazio et al. (2011) observed an increase in RDW in sheep transported 125 km by road for 3 h but no explanation was offered for why this observation occurred. It has been suggested that an increase in RDW can be used as a biomarker to identify pulmonary compromise but the conflicting results presented in these reports suggest that additional studies are needed to investigate this relationship further.

The overall results of this study suggest that long-distance transportation was associated with a significant increase in neutrophil, platelet, monocyte, white blood cell, and red blood cell count after shipping and a decrease in lymphocyte count. Furthermore, MMP-9 complexes, TCO_2 , $\text{TNF}\alpha$, plasma proteins, and SP increased and cortisol concentrations decreased after shipping. Meloxicam treatment reduced the stress neutrophilia and monocytosis after shipping. Circulation cortisol, neutrophil, and basophil concentrations were also inversely proportional to plasma MEL concentrations. Further studies, including examination of the effect of MEL administration on cell function, calf health, and performance, are needed to fully elucidate the overall impact of NSAID administration on

the negative effects of long-distance transportation. These findings may have implications for developing strategies to reduce the impact of transportation stress on the health and performance of feedlot cattle.

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